EurJOC European Journal of Organic Chemistry

FULL PAPER

DOI: 10.1002/ejoc.201500237

The Thia-Michael Reactivity of Zerumbone and Related Cross-Conjugated Dienones: Disentangling Stoichiometry, Regiochemistry, and Addition Mode with an NMR-Spectroscopy-Based Cysteamine Assay

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Dedicated to Professor Iwao Ojima on occasion of his 70th birthday

Keywords: Natural products / Michael addition / Enones / Thiols / Medium-ring compounds / Biological activity / Structure-activity relationships

The cross-conjugated and electrophilic dienone system of the humulane sesquiterpene zerumbone (**1a**) was modified by *E*/*Z* photochemical isomerization and/or by removal of homoconjugation with the isolated endocyclic double bond of the medium-sized ring. The site (C-6/C-9), mode (transient or irreversible), stoichiometry (single or twofold), and comparative rates of thiol addition were evaluated using an NMR-spectroscopy-based cysteamine assay. Dramatic effects were seen, and this highlights the subtleties of the reaction and the limitations of our predictive power in this field. For biological endpoints sensitive to thiol trapping, a substantial separation between Michael reactivity and biological activity was found for **1a** and its analogues. This supports the view that shape complementarity plays a critical role in the covalent binding of Michael acceptors to their macromolecular target(s).

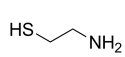
Introduction

Because of concerns over nonspecific toxicity and lack of selectivity, the Michael acceptor motif is rarely introduced into drug leads by design.[1] Paradoxically, our diet is rife with Michael acceptors, and food plants provide countless leads to investigate the biological role of Michael reactivity in molecules that are substantially devoid of toxicity at dietary dosages.[2] The sesquiterpene zerumbone (**1a**) represents an interesting compound to investigate the chemical and biological subtleties of the Michael reaction. Zerumbone is easily available in high yield (up to >4% on a dry-weight basis) from lampuyang (shampoo ginger, *Zingiber zerumbet* Smith), an oriental spice and medicinal plant,[3] and it has been used as a starting material for the semisynthesis of valuable natural products, as well as in diversity-oriented synthesis.[4]

	Δ^2	Δ^6	Δ^9
1a	*E*	*E*	*E*
1b	*E*	*E*	*Z*
1c	*E*	*Z*	*Z*
1d	*Z*	*Z*	*E*
1e	*E*	*Z*	*E*

(*rac*)

	Δ^9
2a	*E*
2b	*Z*



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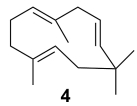

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The covalent interaction of zerumbone with proteins involved in cell proliferation and defence against oxidative/electrophilic stress makes it a potential candidate for cancer

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/ejoc.201500237.

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prevention and treatment.[5] The Michael reactivity of zerumbone is actually surprising, since cross-conjugated cyclohexadienones lack thiol-trapping properties.[6] This suggests a specific contribution from the medium-sized-ring environment. Furthermore, the mode of the thiol addition to zerumbone is also puzzling. In situ NMR spectroscopy experiments have shown that the addition to the disubstituted Δ^9 double bond is transient, reversing upon a solvent switch from DMSO to $CDCl_3$, whereas the trisubstituted Δ^6 double bond reacts in an irreversible way.[6] All Michael reactions are fundamentally reversible and susceptible to transthiolation,[7] but zerumbone adds to the growing number of compounds where this reversibility trait is brought to the extreme, resulting in thiol addition products that are transient, resist isolation, and can be made to undergo retro-Michael reaction by a simple solvent switch or by heating.[8] Remarkably, removal of this transient electrophilic trait leads to a complete loss of biological activity, which suggests that fleeting Michael reactivity can also have biological consequences.[8]

To shed light on this behaviour, and to provide clues of general value for the biological relevance of thiol trapping by electrophilic biologically active agents, we have evaluated the relationship between structure and Michael reactivity in a series of analogues of zerumbone where the cross-conjugated dienone system is modified configurationally and electronically by photochemical isomerization and/or removal of transannular interaction (homoconjugation) with the isolated endocyclic double bond of the medium-sized ring.[9] The cysteamine assay, based on the reaction of thiol-trapping compounds with cysteamine (**3**) in DMSO and phase-switch to $CDCl_3$,[6] was used to obtain information on the stoichiometry and regiochemistry of addition, and on the relative reactivities of the analogues.

Results and Discussion

The configurational isomers of zerumbone (i.e., **1b**–**1d**) were obtained by photochemical isomerization of the natural product. The photochemistry of zerumbone has previously been investigated under rather unusual conditions, namely combining irradiation and thermal isomerization.[10] Under these conditions, the 9*Z* isomer of zerumbone [9-isozerumbone (**1b**)] was formed along with a mixture of products resulting from both thermal and photochemical pericyclic processes. Irradiation of zerumbone at room temperature prevented the formation of the "thermal" photoproducts and cleanly gave 9-isozerumbone (**1b**) as the major reaction product (42%), accompanied by minor amounts of two doubly isomerized analogues, namely 6,9-diisozerumbone (**1c**) and 2,6-diisozerumbone (**1d**). The configurational assignment of the photoisomerized products was based on evaluation of the coupling constant $J_{9,10}$ for the disubstituted (Δ^9) double bond, and on inspection of the ROESY spectra for the two trisubstituted double bonds (Δ^2 and Δ^6). The coupling constant between the vicinal olefin protons 9-H and 10-H was found to have a value of ca. 12 Hz in **1b** and **1c**, consistent with a *Z* configuration, and ca. 16 Hz in **1d** and zerumbone (**1a**), pointing to an *E* configuration. On the other hand, the allylic methyl group at C-7 (Me-13) showed a ROESY cross-peak with 6-H in **1c** and **1d**, while in zerumbone and 9-isozerumbone (**1b**) this methyl group was correlated with the C-5 methylene group. Thus, the cross-conjugated trisubstituted double bond (Δ^6) was *E* in **1a** and **1b**, and *Z* in **1c** and **1d**. In a similar way, the other allylic methyl group (Me-12) showed a ROESY cross-peak with the C-1 methylene group in zerumbone (**1a**), **1b**, and **1c**, which shows an *E* configuration, whereas in **1d** this ROESY cross-peak was replaced by one with 2-H, diagnostic of a *Z* configuration. Based on comparison of ^{1}H NMR spectroscopic data, 2,6-diisozerumbone (**1d**) might correspond to a photoisomer wrongly reported as 6-isozerumbone (**1e**).[10] Taken together, these results show that irradiation can result in the isomerization not only of the two conjugated double bonds, but also of the nonconjugated double bond of zerumbone, presumably because of an antenna effect, where excitation of the dienone chromophore is transmitted to a nearby olefin site, as documented, for instance, in the formation of *lumi*-paclitaxel from the irradiation of paclitaxel.[11] At full conversion, the ratio of photoisomers **1b**/**1c**/**1d** was ca. 7:2:1.

Incomplete degassing and/or oxygen leaking during the irradiation was associated with the formation of variable amounts of epoxides *rac*-**2a**[12] and *rac*-**2b**, resulting in removal of homoconjugation between the cross-conjugated dienone system and the isolated endocyclic double bond. Epoxidation of the isolated double bond had dramatic effects on the chemical shift of the terminal carbon atoms of the dienone system, especially in 9-isozerumbone (**1b**), with a downfield shift of ca. 9 ppm for the C-6 signal, and of ca. 4 ppm for the C-10 signal, suggesting a significant through-space orbital interaction, a typical feature of medium-sized cyclic polyolefins.[9] The yield of the epoxides was increased considerably when the irradiation was carried out under a flow of air; however, this did not result in complete conversion. Despite the presumably radical mechanism of this oxidation, the configurations of the epoxides were the same as those of the products obtained by treating zerumbone (**1a**) and 9-isozerumbone (**1b**) with peracids. The regioselectivity of the epoxidation, with attack occurring at the non-enone double bond, is as expected from consideration of electron density and polarization, but it is nevertheless in contrast to the outcome of the electrophilic addition of bromine to zerumbone, which, surprisingly, occurred chemoselectively at the trisubstituted conjugated dienone double bond.[13] These contrasting observations are presumably related to the lack of planarity of the dienone system of zerumbone. In the solid state, torsion angles with the carbonyl oxygen atom of ca. 45° and 35° were observed for the disubstituted and trisubstituted dienone double bonds, respectively, resulting in a skew arrangement that makes zerumbone chiral and, in principle, resolvable.[14] Pyramidalization and alkyl substitution raise the energy of an olefin HOMO.[15] This effect dominates

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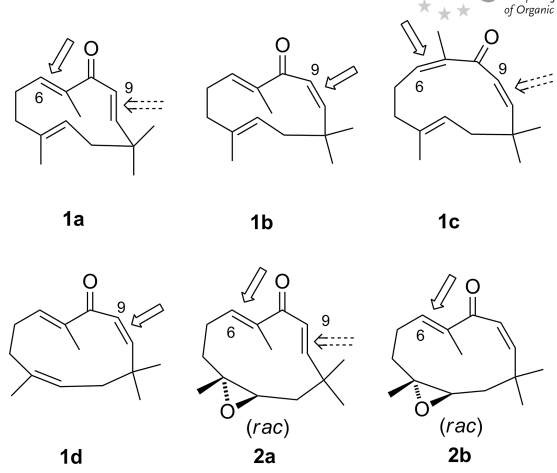
the reaction with a soft electrophile like bromine, but with harder electrophiles like the distal oxygen atom of peracids, the more electron-rich nonconjugated double bond is preferred.

The Michael reactivity of the natural product and its analogues was then investigated with the cysteamine assay.[6] Zerumbone (**1a**) and its derivatives are potentially bifunctional thiol-trapping agents, and the assay was therefore carried out by first treating solutions of the substrates in DMSO with ca. 1.5 equiv. of cysteamine to identify the most reactive site, and next with an excess (4 equiv.) of this reagent to evaluate the overall reactivity pattern. The reversibility was evaluated by using a solvent shift, diluting the DMSO solution of the adducts to 1:10 with $CDCl_3$. In reactions with nitrogen and oxygen nucleophiles, the trisubstituted dienone double bond of zerumbone was reported to be more reactive than the disubstituted double bond,[4] and a similar trend was observed in the NMR-spectroscopy-based cysteamine assay. An excess of the reagent was found to be necessary for the disappearance of the AB system assigned to the Δ^9 double bond. Remarkably, the Michael addition at the Δ^9 disubstituted dienone bond was reversed by the solvent switch, while the one at the other dienone bond (Δ^6) was not.[6] It is not clear how a different degree of pyramidalization and the methyl substitution contribute to this profile of reactivity, but transient thiol addition has also been reported in a strained and partially pyramidalized macrocyclic linear dienone.[16]

Doubly isomerized 6*Z*,9*Z* dienone **1c** showed the same reactivity as the natural product (Scheme 1), but marked differences in reactivity were observed for the other isomers. Thus, 9*Z* isomer **1b** and 2*Z*,6*Z* isomer **1d** behaved as monodentate electrophiles, exclusively giving irreversible monoaddition to the disubstituted Δ^9 double bond, even with a large excess (6 equiv.) of cysteamine. These observations show that isomerization can reverse the reactivity profile of the dienone system in terms of stoichiometry, regiochemistry, and mode of addition. The *gem*-dimethyl substitution at the allylic carbon atom of the disubstituted double bond (C-11) could explain the lower reactivity of this dienone bond compared to the trisubstituted one in zerumbone (**1a**) and its 6*Z*,9*Z* isomer **1c**. However, monoisomerization of the dienone system as in **1b** and **1d** reverses this trend, making the disubstituted double bond more reactive than the trisubstituted one, possibly because of an increased polarization that overrides the steric effect of the allylic *gem*-disubstitution. Remarkably, epoxidation had no effect on the Michael reactivity of zerumbone (**1a**) but dramatically affected that of its 9*Z* isomer **1b**, quenching the thiophilicity of the disubstituted double bond, and leading to the exclusive and irreversible reaction of the trisubstituted dienone bond, even with a large excess (6 equiv.) of cysteamine. These observations highlight the difficulty of predicting the site and stoichiometry of thiol addition in complex electrophilic molecules[7] and undoubtedly provide food for thought for computational studies aimed at providing a mechanistic basis to rationalize this baffling behaviour.



Scheme 1. Stoichiometry, regiochemistry and nature (irreversible/transient) of the Michael addition to zerumbone (**1a**), its stereoisomers **1b**–**1d**, and epoxides **2a** and **2b**. A plain line indicates an irreversible addition, and a dotted line a transient addition.

In comparative experiments in which equimolar amounts of zerumbone (**1a**) and its 9*Z* isomer **1b** were treated with a substoichiometric amount of cysteamine, only the 9*Z* isomer reacted, which shows that isomerization increases the reactivity of the disubstituted dienone double bond. Further competition experiments in which equimolecular pairs of isomers were treated with a substoichiometric amount of cysteamine established the reactivity sequence $9Z > 6E > 6Z > 9E$. In similar competition experiments, the epoxide **2a** of zerumbone and the epoxide **2b** of 9-isozerumbone reacted more slowly than their parent compounds, which suggests that homoconjugation facilitates the interaction with thiols, as would be expected for a lowering of the LUMO energy. Taken together, the results of the cysteamine assay showed that zerumbone and its analogues can react with thiols in different ways. Zerumbone (**1a**) and its 6*Z*,9*Z* isomer **1c** behaved as bifunctional electrophiles, while the other isomers **1d** and **1d** behaved as monofunctional and irreversible electrophiles. Remarkably, the Michael reactivity of the 9*Z* double bond was completely removed by epoxidation of the nonconjugated double bond.

Since Michael reactivity is the hallmark of the biological profile of zerumbone,[5] we were interested in investigating whether, and to what extent, these differences in reactivity translated to differences in biological activity. Thus, the activity of the natural product and its derivatives were investigated in a series of validated biochemical (NF-κB inhibition, Nrf2 activation), and phenotypical [cytotoxicity, ROS (reactive oxygen species) generation] endpoints of zerumbone.[5,17] Consistently with previous reports,[5,17] zerumbone inhibited NF-κB, activated Nrf2, induced ROS, and was toxic to cancer cells. This pleiotropic trait of the natural product was, however, lost in its isomers and derivatives. Thus, epoxides **2a** and **2b** were unable to induce ROS accumulation or inhibit NF-κB, but were still capable to activate the Nrf2 pathway, especially compound **2b**. On the

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other hand, the 9*Z* isomer **1b**, which was significantly less pro-oxidant than **1a**, was cytotoxic, but did not show NF-κB inhibitory activity, despite its higher Michael reactivity than that of the natural product (see the Supporting Information for detailed biological activity data). These observations show that reactivity with thiols and biological activity of these cross-conjugated electrophilic dienones are significantly dissociated. Shape complementarity is therefore critical to biological activity; presumably, this works by positioning the thiol-trapping site in the correct orientation to interact with the reactive cysteine residue(s) of the target molecule(s).

Conclusions

Zerumbone is undoubtedly a rather peculiar electrophilic reagent. Its reactivity profile, as well as its stability compared to that of its deoxy derivative humulene (**4**),[18] might be due to the existence of a troponoid motif resulting from a homoconjugated interaction between the dienone system and the nonconjugated double bond (Figure 1). Nevertheless, we believe that the conclusions of this study provide clues of general relevance. Thus, the puzzling changes observed upon isomerization of the dienone system or removal of homoconjugation highlight how difficult it is to predict reactivity in multifunctional electrophilic agents, while the lack of correlation between biological activity and thiophilicity exemplifies the difficulty of translating test-tube data into the complexity of cell-based experiments.

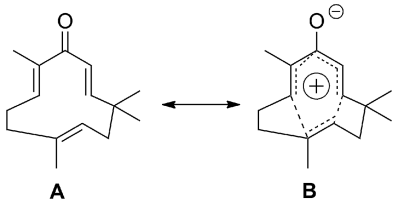
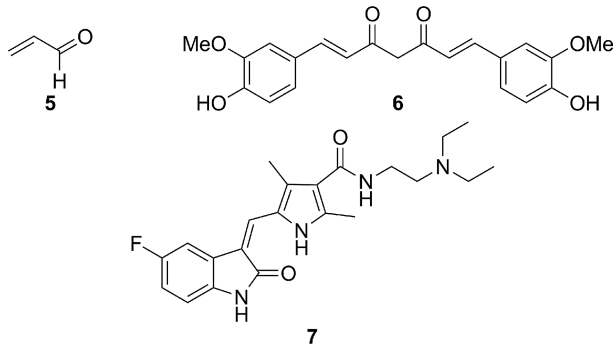


Figure 1. Homoconjugated interaction between the crossed dienone system and the nonconjugated double bond of zerumbone, as expressed by troponoid structure **B**.

Due to a common reactivity-based mechanism of action, compounds like acrolein (**5**), a carcinogenic pollutant, curcumin (**6**), a dietary phenolic, and sunitinib (**7**), an anticancer selective kinase inhibitor, belong, basically, to the same group of thiol-trapping agents. Specificity of action is bound to a "lock-and-dock" strategy,[19] namely the exquisite coupling of a general decrease of reactivity and the possibility of involvement in multicenter engagements capable to accelerate the addition reaction. In this context, the identification of stoichiometry of addition, reactivity center(s) and ranking within a series of analogues are of critical relevance, and we have shown how the cysteamine assay, a quick and unsophisticated test, can provide this information.[20]



Experimental Section

General Remarks: ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were measured with a Varian INOVA spectrometer. Chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H = 7.26 ppm; δ_C = 77.0 ppm). Homonuclear ^{1}H connectivities were determined with COSY experiments. One-bond heteronuclear ^{1}H,^{13}C connectivities were determined with HSQC experiments. Through-space ^{1}H proximities were determined with ROESY experiments with a mixing time of 250 ms. Two- and three-bond ^{1}H,^{13}C connectivities were determined with gradient 2D HMBC experiments optimized for $^{2,3}J$ = 9 Hz. Low- and high-resolution electrospray (ESI) mass spectra were obtained with an LTQ OrbitrapXL (Thermo Scientific) mass spectrometer. Silica gel 60 (70–230 mesh) was purchased from Macherey–Nagel. Reactions were monitored by TLC on Merck 60 F_{254} (0.25 mm) plates, which were visualized by UV inspection and/or staining with H_2SO_4 (5% in ethanol) and heating. The organic phases were dried with Na_2SO_4 before concentration. Zerumbone was isolated from the essential oil of shampoo ginger by direct crystallization,[21] and epoxyzerumbone (**2a**) was prepared according to a literature procedure.[12] The purity (95% or higher) of all of the final products that were evaluated for reactivity and biological activity was assessed by analytical HPLC using isocratic acetonitrile/1% formic acid in water (80:20) as eluent, with UV detection at 240 nm with a JASCO Herculite apparatus equipped with a Phenomenex Luna C18 column.

Photochemical Isomerization of Zerumbone (1a): A solution of zerumbone (**1a**) (1.00 g, 4.5 mmol) in ethanol (100 mL) was degassed by bubbling nitrogen through it for 10 min and then irradiated in a water-cooled immersion-well photochemical reactor by using a 125 W high-pressure mercury lamp. The course of the reaction was monitored by TLC on silica gel (petroleum ether/EtOAc, 9:1), by checking the disappearance of zerumbone (R_f = 0.36) and the formation of the isomerized products [R_f(**1b**) = 0.59; R_f(**1c**) = R_f(**1d**) = 0.42; R_f(**2a**) = R_f(**2b**) = 0.16]. After 2 h, the reaction was complete, and the solution was concentrated. The semisolid residue was purified by gravity column chromatography on silica gel (petroleum ether/EtOAc, 98:2 to 80:20) to give **1b** (410 mg, 42%), a mixture of **1c** and **1d** (180 mg, 18%), and a mixture of epoxides **2a** and **2b** (72 mg, 7%). The mixture of **1c** and **1d** was resolved by preparative HPLC on silica gel (petroleum ether/EtOAc, 95:5) to give **1c** (84 mg) and **1d** (39 mg). Pure samples of epoxides **2a** and **2b** were obtained by epoxidation of zerumbone (**1a**) and 9-isozerumbone (**1b**) rather than by purification of the mixture of these two compounds obtained from the irradiation of **1a**.

(9*Z*)-Isozerumbone (1b): Colourless crystals, m.p. 62–63 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 5.86 (d, J = 12.3 Hz, 1 H, 9-H), 5.78 (m, 1 H, 6-H), 5.34 (d, J = 12.3 Hz, 1 H, 10-H), 5.00 (t, J = 8.8 Hz, 1 H, 2-H), 2.27 (m, 2 H, 5-H_2), 1.96 (m, 1 H, 1a-H), 1.94 (m, 2 H,

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4-H_2), 1.90 (m, 1 H, 1b-H), 1.92 (s, 3 H, 13-H_3), 1.40 (s, 3 H, 12-H_3), 1.07 (s, 6 H, 14-H_3, 15-H_3) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 201.6 (C-8), 151.6 (C-6), 143.6 (C-10), 137.5 (C-7), 134.2 (C-3), 127.3 (C-9), 126.2 (C-2), 41.7 (C-11), 40.8 (C-1), 38.6 (C-4), 33.5 (C-5), 29.0 (C-14), 25.1 (C-15), 10.7 (C-13), 15.6 (C-12) ppm. MS (ESI$^+$): m/z = 241 [M + Na]$^+$. HRMS (ESI$^+$): calcd. for $C_{15}H_{22}ONa$ 241.1568; found 241.1570.

(6*Z*,9*Z*)-Diisozerumbone (1c): Colourless powder, m.p. 75–77 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 6.60 (m, 1 H, 6-H), 5.99 (d, J = 12.2 Hz, 1 H, 9-H), 5.60 (d, J = 12.2 Hz, 1 H, 10-H), 5.18 (t, J = 8.1 Hz, 1 H, 2-H), 2.41 (m, 2 H, 5-H_2), 2.30 (m, 2 H, 4-H_2), 2.05–1.95 (m, 2 H, 1-H_2), 1.75 (s, 3 H, 13-H_3), 1.53 (s, 3 H, 12-H_3), 1.05 (s, 3 H, 14-H_3), 0.83 (s, 3 H, 15-H_3) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 204.8 (C-8), 144.2 (C-10), 137.5 (C-7), 136.2 (C-3), 131.5 (C-6), 126.5 (C-9), 125.0 (C-2), 43.1 (C-11), 41.6 (C-1), 38.2 (C-4), 33.5 (C-5), 29.0 (C-14), 25.1 (C-15), 21.4 (C-13), 19.7 (C-12) ppm. MS (ESI$^+$): m/z = 241 [M + Na]$^+$. HRMS (ESI$^+$): calcd. for $C_{15}H_{22}ONa$ 241.1568; found 241.1572.

(2*Z*,6*Z*)-Diisozerumbone (1d): Colourless powder, m.p. 56–57 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 6.34 (d, J = 16.2 Hz, 1 H, 10-H), 5.88 (d, J = 16.2 Hz, 1 H, 9-H), 5.53 (m, 1 H, 6-H), 5.13 (t, J = 8.1 Hz, 1 H, 2-H), 2.31 (m, 2 H, 4-H_2), 2.18 (m, 2 H, 5-H_2), 2.05–1.91 (m, 2 H, 1-H_2), 1.91 (s, 3 H, 13-H_3), 1.69 (3 s, 3 H, 12-H_3), 1.15 (s, 3 H, 14-H_3), 1.15 (s, 3 H, 15-H_3) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 203.8 (C-8), 161.7 (C-10), 137.5 (C-7), 137.0 (C-3), 131.5 (C-6), 127.5 (C-9), 123.8 (C-2), 42.9 (C-11), 40.1 (C-1), 37.0 (C-4), 33.5 (C-5), 29.3 (C-14), 24.2 (C-15), 21.4 (C-13), 20.7 (C-12) ppm. MS (ESI$^+$): m/z = 241 [M + Na]$^+$. HRMS (ESI$^+$): calcd. for $C_{15}H_{22}ONa$ 241.1568; found 241.1565.

Epoxidation of (9*Z*)-Zerumbone (1b): *m*-Chloroperbenzoic acid (MCPBA; 80%; 84 mg, 0.8 mmol, 2 equiv.) was added to a solution of **1b** (85 mg, 0.4 mmol) in CH_2Cl_2 (2 mL). The mixture was stirred at room temp. for 15 min, then it was diluted with CH_2Cl_2 (10 mL), and worked up by concentration. The residue was purified by gravity column chromatography on neutral alumina (10 g; petroleum ether/EtOAc, 8:2) to give **2b** (80 mg, 93%).

(9*Z*)-Isozerumbone Oxide (2b): Colourless foam. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 6.04 (d, J = 11.8 Hz, 1 H, 10-H), 5.98 (br. t, 1 H, 6-H), 5.43 (d, J = 11.8 Hz, 9-H), 2.87 (dd, J = 7.9, 3.7 Hz, 1 H, 2-H), 2.85 (m, 1 H, 4a-H), 2.20 (m, 2 H, 5-H_2), 1.91 (s, 3 H, 13-H_3), 1.82 (dd, J = 13.2, 3.7 Hz, 1 H, 1a-H), 1.25 (s, 3 H, 3 H, 12-H_3), 1.18 (m, 2 H, 4b-H, 1b-H), 1.10 (s, 6 H, 14-H_3, 15-H_3) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 202.8 (C-8), 159.6 (C-6), 147.9 (C-10), 139.5 (C-7), 128.4 (C-9), 62.9 (C-2), 61.5 (C-3), 42.7 (C-4), 38.2 (C-1), 36.1 (C-5), 29.8 (C-14), 24.8 (C-11), 24.0 (C-15), 15.6 (C-13), 12.2 (C-12) ppm. MS (ESI$^+$): m/z = 257 [M + Na]$^+$. HRMS (ESI$^+$): calcd. for $C_{15}H_{22}O_2Na$ 257.3238; found 257.3244.

Cysteamine Assay: In a standard 5 mm NMR tube (Armar Chemicals), the substrate (ca. 5 mg) was dissolved in [D_6]DMSO (500 μL), and the NMR spectrum was recorded. Cysteamine (1.5 equiv.) was then added, and the spectrum was recorded again 5 min after the addition. A second aliquot (4 equiv.) of cysteamine was added, and the spectrum was registered again. An aliquot (25 μL) of the solution was then transferred into a second NMR tube containing $CDCl_3$ (500 μL), and a new spectrum was recorded. A positive assay was evidenced by the disappearance of the dienone signals of the substrate, and the reversibility of the Michael addition by the reappearance of some of them after dilution to 1:20 with $CDCl_3$. Comparative reactivity experiments were carried out by treating roughly equimolar amounts of two substrates with a substoichiometric amount of cysteamine (0.8–1.0 equiv. based on a single reactive site in only one of the two substrates).

Cytotoxicity Assays: SK-S-NH cells were seeded at a density of 104 cells/well in 96-well plates (200 μL), and were incubated with increasing concentrations of the compounds for 24 h. After that, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); 5 mg/mL; 100 μL] from a mixed solution of MTT and DMEM (Dulbecco's modified Eagle medium) (1:2) was added to each well, and cells were incubated at 37 °C in the dark for 4 h. Then the reaction was stopped, the supernatant was removed, DMSO (100 μL) was added to each well, and the wells were incubated for 10 min with gentle shaking. Finally, the absorbance was measured at 550 nm by using a TriStar LB 941 instrument (Berthold Technologies). The absorbance of untreated cells was taken as 100% viability, and the percentage of cellular viability was calculated.

NF-κB and Nrf2 Transcriptional Assays: Anti-NF-κB (NF = nuclear factor) activity was investigated in 5.1 cells, a validated "in vitro" model for the study of TNFα-induced NF-κB activation (TNF = tumor necrosis factor).[22] The 5.1 cells were stimulated with TNFα (20 ng/mL) in the presence or absence of the compounds for 6 h. For the activation of the antioxidant response element (ARE), SK-*N*-SH cells were transiently transfected with the pGL3-ARE-Luc plasmid, which contains the Nqo1 antioxidant response element fused to the luciferase reporter gene. After 24 h, the cells were stimulated with the compounds for 6 h. After stimulation, the cells were washed twice in PBS (phosphate-buffered saline) and lysed in tris-phosphate pH = 7.8 (25 mM), $MgCl_2$ (8 mM), DTT (1 mM), Triton X-100 (1%), and glycerol (7%) at room temperature in a horizontal shaker for 15 min. After centrifugation, the supernatants were used to measure luciferase activity by using an Autolumat LB 9510 instrument (Berthold) following the instructions of the luciferase assay kit (Promega, Madison, WI, USA). The protein concentration in the cell extracts was measured by the Bradford method (BioRad, Hercules, CA, USA). The RLU/μg (RLU = relative light units) was calculated, and the results were expressed as percentage of inhibition of NF-κB activity induced by TNFα (100% activation). For Nrf2 activation, the specific ARE-Luc induction was expressed as induction relative to the control (untreated cells).

ROS Determination: To evaluate the induction of ROS, SK-N-SH cells were treated with increasing concentrations of compounds for 12 h. Then the cells were incubated in PBS with DiOC6(3) (green fluorescence; 20 nM; Molecular Probes, Eugene, OR, USA) at 37 °C for 20 min, followed by analysis with a FACS Canto II flow cytometer (Beckton Dickinson, NJ, USA).

Statistical Analysis: Data are expressed as mean ± SD. Differences were analysed by the Student's t test. $P < 0.05$ was considered significant.

Acknowledgments

We thank Dr. Daniel Joulain for a generous gift of zerumbone, and Mr. Andry Leeman (Mensa Group, Jakarta, Indonesia) for providing fresh zerumbet roots. We are grateful to Ministero dell'Università e Ricerca Scientifica e Tecnologica (MURST), Italy for financial support (Project 2009RMW3Z5: "Metodologie sintetiche per la generazione di diversità molecolare di rilevanza biologica"). Mass and NMR spectra were recorded at the "Centro di Servizio Interdipartimentale di Analisi Strumentale", Università di Napoli "Federico II".

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Received: February 18, 2015
Published Online: ■

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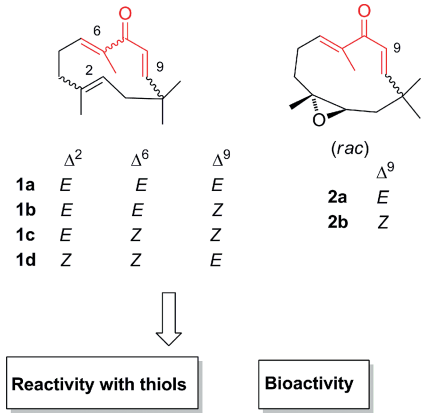
EurJOC European Journal of Organic Chemistry

Natural Products

Using the NMR-spectroscopy-based cysteamine assay, the thia-Michael reactivity of the cross-conjugated dienone zerumbone and its photoisomers and epoxides was investigated, and marked differences were found. For biological endpoints sensitive to Michael acceptors, a substantial separation between reactivity and biological activity was observed.



Keywords: Natural products / Michael addition / Enones / Thiols / Medium-ring compounds / Biological activity / Structure-activity relationships

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